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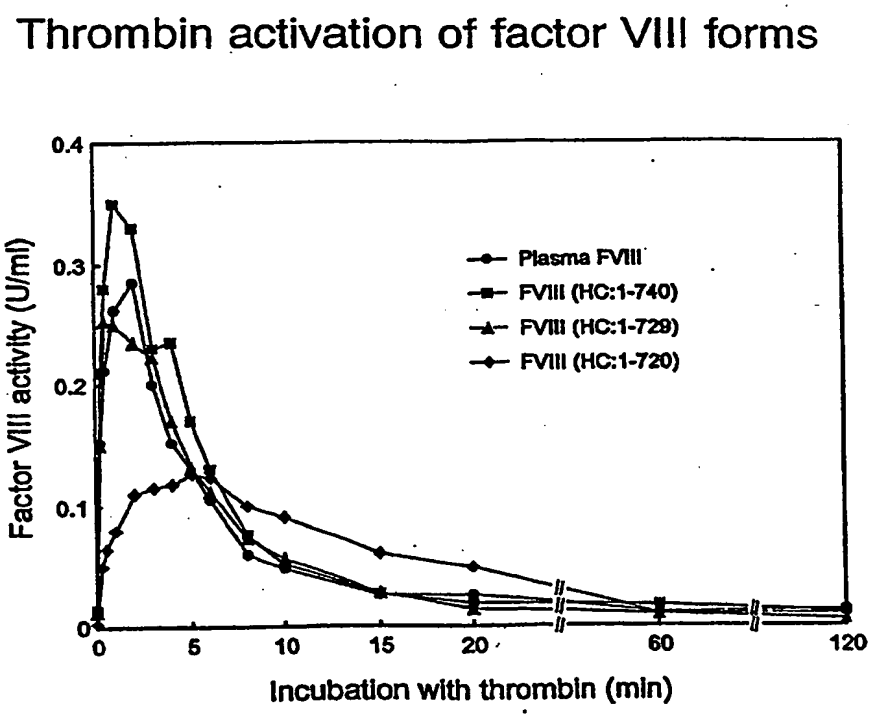
(54) Title: NEW FACTOR VIII POLYPEPTIDES

(57) Abstract

A factor VIII polypeptide comprising a heavy chain having an amino acid sequence corresponding to the amino acid sequence of the N-terminal part of full length Factor VIII and a light chain having an amino acid sequence corresponding to the amino acid sequence of the C-terminal part of full length Factor VIII wherein the heavy chain is shorter than the A1-A2 domain of full length Factor VIII exhibits the coagulating effect of Factor VIII and may be used for preventing or treating diseases caused by absence or deficiency of Factor VIII in a subject.



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TITLE

New Factor VIII polypeptides.

FIELD OF THE INVENTION

The present invention relates to new Factor VIII polypeptides showing coagulant activity, a method for the preparation thereof, pharmaceutical preparations comprising the new Factor VIII polypeptides, the use of these polypeptides for the preparation of a pharmaceutical preparation especially for the treatment of diseases caused by an absence or deficiency of the Factor VIII of a subject.

BACKGROUND OF THE INVENTION

Haemophilia A is an X-chromosome-linked inherited disease which afflicts 1-2 males per 10,000. The desease is caused by an absence or deficiency of Factor VIII. Factor VIII is a large glycoprotein (native M_r 330000 - 360000), which is present in plasma at low concentrations (0.1 nM (S.I. Rapaport, West. J. Med. (1993) 158:153-161). It is an essential element in the proteolytic cascade which converts soluble fibrinogen to insoluble fibrin, forming a clot to prevent blood loss from traumatized tissue. In the bloodstream, it is found in noncovalent association with von Willebrand Factor (vWF) which acts as a stabilizing carrier protein. Factor VIII is susceptible to cleavage by thrombin, activated protein C, plasmin, and other serine proteases. It is generally isolated from plasma as a series of related polypeptides ranging from M_r 160000-40000 with predominant species of M_r 92000 (the heavy chain) and M_r 80000 (the light chain). This complex pattern has made the analysis of the structure of active Factor VIII very difficult.

Factor VIII and the related polypeptides have been described by F. Rotblat et al, Biochemistry (1985) 24:4294-4300; G.A. Vehar et al, Nature (1984) 312:337-342; J.J. Toole et al, Nature (1984) 312:342-347; and M.A. Truett et al, DNA (1985) 4:333-349. The sequence has been reported by J.J. Toole et al, supra; W.I. Wood et al, Nature (1984) 312:330-336; and M.A. Truett et al, supra.

The full-length protein contains three repeats of the A-domain and two repeats of the C-domain together with a heavily glycosylated B-domain, ordered A1-A2-B (the heavy chain) and A3-C1-C2 (the light chain). The B-domain is not required for the function of Factor VIII (Burke et al. (1986) J.Biol.Chem. 261:12574-12578).

By thrombin activation, the heavy chain is cleaved between the A1 and the A2-domains and between the A2 and B domains, and 41 amino acids is cleaved off from the N-terminus of the light chain.

Factor VIII has historically been isolated from blood in a concentrated form for therapeutic treatment of haemophilia. However, Factor VIII is only present in the blood in extremely small amounts and a vast number of donors have to be involved and the isolation and purification process which is, moreover, laborious and expensive. Concerns regarding transmission of HIV and other blood-borne diseases as well as shortage of supplies have especially stimulated activity to provide alternative supplies of Factor VIII, thus leading to the development of recombinant techniques.

The preparation of proteins having Factor VIII activity by recombinant techniques has inter alia been disclosed in a number of patent publications. Thus, European Patent Application No. 160 457 and International Patent Application No. WO 86/01961 disclose recombinant production of full length Factor VIII, European Patent Application No. EP 150 735 discloses a complex of subunits of Factor VIII having coagulant activity and recombinant production of subunits of Factor VIII, European Patent Application No. EP 232 112 and International Patent Application

No. WO 91/07490 disclose co-expression of subunits of Factor VIII for the production of complexes showing coagulant activity, and International Patent Application No. WO 86/06101, International Patent Application No. WO 87/04187, International Patent Application No. WO 87/07144, International Patent Application No. WO 88/00381, European Patent Application No. EP 251 843, European Patent Application No. EP 253 455, European Patent Application No. EP 254 076, U.S. Patent No. 4.980.456, European Patent Application No. EP 294 910, European Patent Application No. EP 265 778, European Patent Application No. EP 303 540, and International Patent Application No. WO 91/09122 disclose recombinant expression of Factor VIII having one or more deletions in the molecule, or binding to antibodies inhibiting Factor VIII.

It is advantageous to express shortened forms of Factor VIII (as compared to the full length molecule) as it is difficult to reach an acceptable level of production due to low expression levels and instability of the expressed product during expression and purification.

Furthermore, it is desirable to identify the smallest fraction of full length Factor VIII showing coagulant activity as such a shortened form may also be used for treating inhibitor patients having developed antibodies against epitopes in the C-terminal part of the heavy chain.

WO 87/07144 discloses the preparation of deletion analogues of Factor VIII lacking 1-1317 amino acid residues from Ser-373 through Arg-1689. However, no examples discloses the preparation of analogues comprising a shortened A2 domain of Factor VIII, and no results are present showing coagulant activity for Factor VIII analogues comprising a shortened A2 domain.

British Journal of Haematology 1993 (85), 133-142 discloses the preparation of deletion analogues of Factor VIII lacking amino acid residues 713 through 1637.

Such analogues show no activity in clotting assay but do show activity in a Factor Xa-generating assay.

BRIEF DESCRIPTION OF THE INVENTION

It has surprisingly been found that new shortened forms of Factor VIII lacking a part of the A2 domain of the heavy chain exhibits coagulant activity up to the same level as the complex of the full A1-A2 and A3-C1-C2 domains.

Thus, in a first aspect, the invention relates to a Factor VIII polypeptide comprising a heavy chain lacking a part of the C terminal part of the A2 domain.

In a second aspect, the invention relates to a method for the preparation of a Factor VIII polypeptide comprising a heavy chain lacking a part of the C terminal part of the A2 domain.

In a further aspect, the invention relates to a pharmaceutical preparation comprising a Factor VIII polypeptide comprising a heavy chain where a part of it or all is lacking a part of the C terminal part of the A2 domain.

In a still further aspect, the invention relates to the use of a Factor VIII polypeptide comprising a heavy chain lacking a part of the C terminal part of the A2 domain for the preparation of a pharmaceutical preparation for the prevention or treatment of diseases caused by absence or deficiency of Factor VIII in a subject.

In accordance with another aspect, the invention relates to a method for preventing or treating diseases caused by absence or deficiency of Factor VIII.

In yet another aspect, the invention relates to a method of preparing a pharmaceutical preparation comprising a Factor VIII polypeptide of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is further explained with reference to the drawings on which

Fig. 1 shows an SDS-PAGE of Factor VIII polypeptides of the invention as compared to Factor VIII polypeptides, containing the complete A2-domain,

Fig. 2 shows RP-HPLC profiles of LysC peptides from the heavy chain of Factor VIII polypeptides of the invention as compared to the heavy chain, containing the complete A2-domain,

Fig. 3 shows RP-HPLC profiles of AspN peptides from the heavy chain of Factor VIII polypeptides of the invention as compared to the heavy chain of Factor VIII polypeptides containing the complete A2-domain,

Fig. 4 shows the inhibition of Factor VIII activity by a monoclonal antibody for peptides of the invention as compared to Factor VIII polypeptides containing the complete A2-domain and plasma Factor VIII,

Fig. 5 shows a time-study of thrombin activation of FVIII polypeptides as measured by SDS-PAGE,

Fig. 6 shows a time-study of thrombin activation of FVIII polypeptides as measured by a clotting assay.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to new Factor VIII polypeptide comprising a heavy chain having an amino acid sequences corresponding to the amino acid

sequence of the N-terminal part of full length Factor VIII and a light chain having an amino acid sequence corresponding to the amino acid sequence of the C-terminal part of full length Factor VIII wherein the heavy chain is shorter than the A1-A2 domain of full length Factor VIII. The heavy and light chains are preferably bridged via a metal ion bridge. Such a bridge is suitably formed via a divalent ion such as Mn^{2+}, Ca^{2+} or Co^{2+}. Preferably the bridge is a calcium bridge.

The Factor VIII polypeptides of the invention prefererably lacks a part of the C-terminal part of the A2 domain. Preferred Factor VIII polypeptides of the invention comprises a heavy chain comprising the amino acid residues 1-720 or 1-729 of the heavy chain of full length Factor VIII.

It has surprisingly been found that the Factor VIII polypeptide of the invention comprising a heavy chain comprising amino acid residues 1-729 of the full length Factor VIII exhibits a coagulant activity of the same level as Factor VIII polypeptide comprising the full heavy chain.

It has also surprisingly been found that the Factor VIII polypeptide of the invention comprising a heavy chain comprising amino acid residues 1-720 of full length Factor VIII exhibits a specific activity as measured in a chromogenic assay of the same level as Factor VIII polypeptides comprising full heavy chain and a specific activity as measured in a clot assay of about 50%.

The Factor VIII polypeptides of the invention normally will comprise a light chain having an amino acid sequence corresponding to amino acids 1649-2332 of the C terminal of full length Factor VIII.

In the alternative, the Factor VIII polypeptides comprise a light chain having an amino acid sequence corresponding to amino acids 1690-2332 of the C terminus full length Factor VIII.

The invention further relates to a method for preparing a Factor VIII polypeptide comprising a heavy chain having an amino acid sequence corresponding to the amino acid sequence of the N-terminal part of full length Factor VIII and a light chain having an amino acid sequence corresponding to the amino acid sequence of the C-terminal part of full length Factor VIII wherein the heavy chain shorter than the A1-A2 domain of full length Factor VIII wherein a Factor VIII polypeptide comprising the full A1-A2 domain of full length Factor VIII is treated with a protease cleaving off the C-terminal part of the A2 domain.

The Factor VIII polypeptides of the invention may be prepared starting from a Factor VIII polypeptide isolated from plasma by methods known per se, e.g. as described in EP patent No. 83483, EP patent No. 150735 or EP patent No. 197901 or produced by recombinant techniques, e.g. as described in the patent applications listed above.

In a preferred embodiment of the invention the Factor VIII polypeptides of the invention are prepared by coexpession of the heavy and light chains of Factor VIII as disclosed in WO91/07490. Such Factor VIII polypeptides lack the B domain of full length Factor VIII and comprise a heavy chain metal ion-bridged to a light chain showing coagulant activity. The Factor VIII polypeptides of the invention may be generated by proteolytic digestion in the medium.

The Factor VIII polypeptides of the invention may be purified and isolated by methods known per se for purification and isolation of Factor VIII polypeptides.

The invention also relates to a pharmaceutical preparation comprising a Factor VIII polypeptide comprising a heavy chain having an amino acid sequences corresponding to the amino acid sequence of the N-terminal part of full length Factor VIII and a light chain having an amino acid sequence corresponding to the amino acid sequence of the C-terminal part of full length Factor VIII wherein all or

a part of the Factor VIII has a heavy chain shorter than the A1-A2 domains of full length Factor VIII in admixture with a parenterally acceptable vehicle or excipient.

Furthermore, the invention relates to the use of a Factor VIII polypeptide comprising a heavy chain having an amino acid sequences corresponding to the amino acid sequence of the N-terminal part of full length Factor VIII and a light chain having an amino acid sequence corresponding to the amino acid sequence of the C-terminal part of full length Factor VIII wherein the heavy chain is shorter than the A1-A2 domains of full length Factor VIII for the preparation of a pharmaceutical preparation.

Preferably the Factor VIII polypeptides of the invention are used for the preparation of a pharmaceutical preparation for the prevention or treatment of diseases caused by absence or deficiency of Factor VIII in a subject.

The invention also relates to a method for preventing or treating diseases caused by absence or deficiency of Factor VIII in a subject comprising administering to the subject a pharmaceutically active amount of a Factor VIII polypeptide comprising a heavy chain having an amino acid sequences corresponding to the amino acid sequence of the N-terminal part of full length Factor VIII and a light chain having an amino acid sequence corresponding to the amino acid sequence of the C-terminal part of full length Factor VIII wherein all or a part of the Factor VIII has a heavy chain shorter than the A1-A2 domains of full length Factor VIII in admixture with a pharmaceutically acceptable vehicle or excipient.

In a further aspect, the invention relates to a method of preparing a pharmaceutical preparation comprising a Factor VIII polypeptide comprising a heavy chain having an amino acid sequences corresponding to the amino acid sequence of the N-terminal part of full length Factor VIII and a light chain having an amino acid sequence corresponding to the amino acid sequence of the C-terminal part of full length Factor VIII wherein all or a part of the Factor VIII has a

heavy chain shorter than the A1-A2 domains of full length Factor VIII with pharmaceutically acceptable vehicle and/or excipients and forming a suitable dosis form of the pharmaceutical preparation.

As used herein the term "full length Factor VIII" designates the full molecule comprising the amino acid residues 1-2332 as disclosed in Nature (1984) 312:339.

As used herein the term "heavy chain" or "HC" designates the A1-A2 repeats of Factor VIII comprising the amino acid residues 1-740 of full length Factor VIII as disclosed in Nature (1984) 312:341.

The term "light chain" or "LC" as used herein designates the A3-C1-C2 repeats of Factor VIII comprising the amino acid residues 1649-2332 as disclosed in Nature (1984) 312:341.

EXPERIMENTAL PART

Summary

Recombinant Factor VIII lacking the B domain was purified by a procedure including an affinity chromatography step using a monoclonal antibody directed against the C-terminal part of the heavy chain. A part of the Factor VIII did not bind to the column, due to C-terminal truncation of the A2-domain of the heavy chains. Two Factor VIII forms were purified from the fractions not bound to the antibody column. By peptide mapping and isolation of C-terminal peptides by affinity chromatography followed by amino acid sequencing and mass spectrometry, it is shown, that the two C-terminal truncated forms of Factor VIII contains heavy chains consisting of amino acids 1-720 (FVIII(HC:1-720)) and 1-729 (FVIII(HC:1-729)), respectively, while the Factor VIII bound to the antibody column

contains a heavy chain consisting of amino acid 1-740 corresponding to the entire A1 and A2-domains. FVIII(HC:1-729) have the same specific activity as FVIII(HC:1-740), and is activated by thrombin at a similar rate. FVIII(HC:1-720) have the same specific activity when the activity is measured in a chromogenic assay, however, the specific activity is a factor two lower when the specific activity is measured in a clotting assay. Similary, FVIII(HC:1-720) is activated by thrombin at a slower rate and to a lower level compared with FVIII(HC:1-740), FVIII(HC:1-729), and plasma Factor VIII.

Materials and Methods

Purification of Factor VIII

Conditioned medium comprising recombinant Factor VIII in the form of a complex of the M_r 90000 and M_r 80000 subunits of Factor VIII joined by a calcium bridge prepared as disclosed in WO 91/07490 containing 20 U Factor VIII pr. ml was filtrated. The filtrate was applied to a cation-exchange S-F (Pharmacia LKB) column and eluted using a salt gradient (increasing the ionic strength).

The eluate from the column was loaded on an immunoaffinity column consisting of an antibody (F25-IgG) directed against the C-terminal part of the heavy chain coupled to CNBr activated Sepharose 4B (Pharmacia) equilibrated with 50 mM TrisCl pH 7.3 containing 150 mM NaCl, 10 mM $CaCl_2$, 10% (v/v) glycerol and 0.02% (v/v) Tween 80 at room temperature. The column was washed with 6 volumes of starting buffer. The flow-through containing FVIII(HC:1-729) and FVIII(HC:1-720) was collected. The column was washed further with 4 volumes 50 mM TrisCl pH 7.3 containing 0.65 M NaCl before eluting with 2.5 volumes of 20 mM TrisCl pH 7.3 containing 2.5 M NaCl, 50% (v/v) ethylenglycol, 10 mM $CaCl_2$ and 0.02% Tween 80. The eluate containing FVIII(HC:1-740) were desalted on a Sephadex G25 column (5.3 x 32 cm, Pharmacia).

The F25-IgG was prepared by purifying Factor VIII HC from plasma as described in WO 88/00210. Using this isolated Factor VIII HC the monoclonal antibody (F25-IgG) was prepared using the procedure disclosed in Thromb. Haemostas 1985:54, 586-590.

The Factor VIII forms were finally purified on a MonoQ PC 1.6/5 column using the SMART system (Pharmacia). Approximately 800 U FVIII(HC:1-740) or FVIII(HC:1-729) and FVIII(HC:1-720) was loaded on the column equilibrated with 20 mM TrisCl pH 7.5 containng 150 mM NaCl, 10 mM $CaCl_2$, 10% glycerol (v/v) and 0.02% (v/v) Tween 80 at room temperature at a flow of 100 μl min^{-1}. After washing, a 30 min gradient of 150 to 500 mM NaCl in the buffer was applied.

The heavy chain forms were separated from the complex and isolated as described in Thromb. Haemostas. 1987:58, 1043-1048.

Enzymatic cleavage

Reduced and amidomethylated heavy chains from FVIII(HC:1-740) (570 pmol), FVIII(HC:1-729) (460 pmol), and FVIII(HC:1-720) (40 pmol) were incubated with LysC endopeptidase (Boehringer Mannheim) at a LysC endopeptidase to heavy chain ratio of 1:50 (w/w) for four hours at 37°C in 0.2 M ammonium bicarbonate. The reaction was stopped by adding 10% triflouroacetic acid to pH 2.

For anhydrotrypsin affinity purification approximately 4 nmol heavy chain from FVIII(HC:1-720) was reduced and alkylated, and cleaved with 3.1 μg LysC endoprotease as described. The reaction was stopped by adding PMSF (Phenylmethane sulfonyl-fluoride) to a final concentration of 1 mM.

AspN endoprotease digestions were made on heavy chains from FVIII(HC:1-740) (approximately 1.6 nmol), FVIII(HC:1-729) (approximately 0.9 nmol), and FVIII-

(HC:1-720) (approximately 0.2 nmol). After redissolvation in 8 M urea, 0.75 μg AspN endoprotease (Boehringer Mannheim) in 10 mM TrisCl pH 7.5 was added to each sample to a final concentration of urea of 1 M. After 1 hour at 22°C another 0.75 μg AspN endoprotease was added to each sample and the reactions were continued for 15 hours before stopping by adding 10% trifluoro-acetic acid to pH 2.

For time-study of thrombin cleavage of Factor VIII forms measured by SDS-PAGE plasma Factor VIII, FVIII(HC:1-740), FVIII(HC:1-729), and FVIII(HC:1-720) were diluted into 1.25 ml of 20 mM TrisCl pH 7.5 containing 150 mM NaCl, 10 mM $CaCl_2$, 10% (v/v) glycerol and 0.02% (v/v) Tween 80 to a final concentration of 20 U/ml. The samples were preincubated 2 min at 37°C before a 100 μl sample was withdrawn an added to 20 μl icecold 50% (w/w) trichloracetic acid containing 0.2% (w/w) sodiumdeoxycolat. Human α-thrombin (Boehringer Mannheim) was added to the remaining Factor VIII to a final concentration of 0.1 U/ml, and 100 μl samples were withdrawn at the times indicated in Fig. 5 and the cleavage stopped as described above. The samples were incubated 30 min at 4°C before centrifugation 10 min at 18000 x g, and the precipitate analyzed by SDS-PAGE.

When the time-study is measured by a clotting assay, the Factor VIII concentration was 0.9 U/ml, and the final volume 0.8 ml. Samples of 50 μl were withdrawn at times indicated on Fig. 6 and analyzed by a clotting assay on Amelung Coagulometer as described below.

For thrombin cleavage of samples to SDS-PAGE (Fig. 1) 2 U Factor VIII were diluted to 100 μl in 20 mM TrisCl pH 7.5 containing 150 mM NaCl, 10 mM $CaCl_2$, 10% (v/v) glycerol and 0.02% (v/v) Tween 80 and incubated for one hour at 37°C with 0.5 U/ml human α-thrombin (Boehringer Mannheim). The Factor VIII was precipitated by adding 20 μl of icecold 50% (w/w) trichloracetic acid containing 0.2% (w/w) sodiumdeoxycolat as described above.

Affinity purification of C-terminal peptides

The LysC endoprotease peptides from FVIII(HC:1-720) heavy chain was added to 1 ml anhydrotrypsin agarose (Clontec) packed in a BioRad column with a diametre of 0.9 cm equilibrated with 50 mM sodium acetate pH 5.0 containing 20 mM $CaCl_2$ at a flow of 0.18 ml min^{-1}. The column was washed with 20 volumes of starting buffer and fractions of 0.5 ml collected. Absorbance at 227 nm was detected. The bound peptides were eluted with 10 volumes 5 mM HCl pH 2.5. The pH of the pooled fractions containing the non-bounded peptides was adjusted to pH 2 by adding 10% trifluoroacetic acid before rechromatography on reverse phase-HPLC. A sample containing only LysC endoprotease and buffer was runned in parallel.

Reverse phase-HPLC

All digests and pooled fractions from the anhydrotrypsin agarose column were analyzed by reverse phase-HPLC (Applied Biosystems model 130A) using a Brownlee C18 column (2.1 x 220 mm, Applied Biosystems) and a 50 min linear gradient from 3.5 to 50.4% acetonitrile in 0.1% trifluoroacetic acid. The flow rate was 275 μl min^{-1}. Effluents were monitored at 214 nm, and fractions collected manually.

Sequence, mass and amino acid analyses

The peptides (10-100 pmol) were sequenced on an Applied Biosystem model 477A sequencer equipped with on-line model 120A HPLC using standard programs as described by the manufacturer.

Mass spectra were recorded with a time-of-flight plasma desorption mass spectrometre (Bio-Ion 20, Applied Biosystem) at 16 kV acceleration.

Samples for amino acid analyses were hydrolyzed for 20 hours under vacuum at 110°C in 6 M HCl containing 0.1% phenol and 0.1% dithiodipropionic acid (Barkholt and Jensen (1989) Anal. Biochem. 177: 318-322). For determination of specific activity 2-3 hydrolyses of each sample were made. The samples were acetone precipitated and redissolved in H_2O to approximately 0.1 $\mu g\ \mu l^{-1}$. Norleusine was added to each sample (1.5 nmol each) as internal standard. The samples were dryed in a speed-vac, and hydrolyses performed as decsribed above.

Activity determination

Chromogenic Assay

The activity of Factor VIII was measured in a chromogenic assay (Coatest, Cromogenix), as described by the manufacturers, except that all reactions were carried out at room temperature and that the incubation times were altered: Phospholipid, Factor IXa + Factor X, $CaCl_2$ and the diluted sample was incubated 15 min before adding the substrate + the thrombin inhibitor, and the colour reaction was allowed to take place for 10 min.

Clotting Activity

Clotting activity of Factor VIII was measured as the ability to restore clotting activity of FVIII deficient plasma (ACL analyses, IL Laboratories, or Amelung coagulometer, Pharmacia).

Clotting time analysis on the ACL instrument was carried out as described by the manufacturer. All reagens were from IL Laboratories except Factor VIII deficient plasma and APTT reagens that were from Organon Teknika. For clotting time analysis on the Amelung instrument, 50 µl Factor VIII deficient plasma, 20 µl APTT reagens (both from Organon Teknika) and 80 µl 20 mM TrisCl pH 7.5 containing 150 mM NaCl was incubated 5 to 10 min at 37°C before the sample was added and clotting-time measured. In all cases activities were calculated by comparing with the activity of a plasma standard calibrated against a WHO standard or by using a partially purified Factor VIII standard with known activity.

Electrophoresis

SDS-PAGE was performed on reduced samples in 7.5% polyacrylamide gels as described in Biochemistry (1991) 30:1533-1537 using the BioRad Mini-Protean system. The gels were silver stained.

Results

Purification of recombinant Factor VIII forms

FVIII(HC:1-740), FVIII(HC:1-729) and FVIII(HC:1-720) together with heavy chains from the three Factor VIII forms were purified as described in Methods. Fig. 1 shows SDS-PAGE of the three Factor VIII forms and thrombin generated fragments from theese compared with plasma Factor VIII containing no B-domain. FVIII(HC:1-740) have similar Mr as the plasma Factor VIII, while FVIII(HC:1-729) and FVIII(HC:1-720) had slightly lower Mr of the heavy chains. The Mr differences is located in the A2-domains as seen by the Mr of the fragments generated by thrombin cleavage (see arrows on Fig. 1).

Peptide mapping of Factor VIII forms

Identification of the differences of the Factor VIII forms was done by LysC and AspN peptide mapping of the isolated heavy chains on reverse phase-HPLC. Fig. 2 shows the LysC peptide maps of reduced and alkylated heavy chain from the recombinant Factor VIII forms. Peaks not seen in all three maps were analyzed by amino acid sequencing and mass spectrometry (see Table I below).

Table I. Amino acid sequences and masses of LysC peptides from the heavy chains marked on Fig. 2. Observed M_r (Obs.) are the masses determined by mass spectrometry. Calculated M_r (Cal.) are deduced from the cDNA sequence. The amino acid numbering (AA no.) corresponds to the mature Factor VIII heavy chain. Small letters indicate tentative sequence assignments. ND = not determined.

Sample	Peptide	AA no.	Sequence	M_r Obs.	M_r Cal.
FVIII(HC:1-740)	A	734-740	NNAIEPR	814.4	813.9
FVIII(HC:1-740)	B	714-733	NTGDYYEDSY-EDISaYLLSK	ND	2347.4
		214-230	NSLMQDRDAA-SARAWPK	ND	1918.1
FVIII(HC:1-740)	C	714-733	NTGDYYEDSY-EDISAYLLSK	2348.3	2347.4
FVIII(HC:1-729)	D	714-729	NTGDYYEDSY-EDISAY	-	1905.9
FVIII(HC:1-729)	E	714-720?	ntgDYyx	-	861.8
FVIII(HC:1-720)	E	714-720	NTGDYYE	-	861.8

The peptide marked A, corresponding to amino acid 734-740, was only present in FVIII(HC:1-740), showing that only this form contains the full-length A2-domain. Only FVIII(HC:1-729) contained the peak marked D, which corresponds to amino

acid 714-729. Because LysC is not cleaving peptides C-terminal to Tyr, this indicates that FVIII(HC:1-729) have C-terminus at Tyr729. Both FVIII(HC:1-729) and FVIII(HC:1-720) contains the peaks marked E corresponding to amino acid 714-720. This peak is not seen in FVIII(HC:1-740) indicating that some Factor VIII have C-terminus at Glu720. The prescence of peak E in FVIII(HC:1-729) could be due to copurification of some FVIII(HC:1-720) in the FVIII(HC:1-729) preparation. FVIII-(HC:1-740) contains the peaks marked B and C both containing amino acid 714-733. The prescence of this peptide in two peaks could be due to partial sulfatation of one or more of the expected sulfate groups at Tyr 118, Tyr 119 and 723 as seen in the full-length FVIII molecule expressed in Chinese Hamster ovary cells (Mikkelsen et al., Biochemistry (1991), 30, 1533-1537.

AspN peptide mapping of the unreduced heavy chains from FVIII(HC:1-740), FVIII(HC:1-729), and FVIII(HC:1-720) is shown in Fig. 3. Table II shows amino acid sequences and mass spectrometry data of the peaks deviating in retention time among the three maps.

Table II. Amino acid sequences and masses of AspN peptides from the heavy chains marked on Fig. 3. Observed M_r (Obs.) are the masses determined by mass spectrometry. Calculated M_r (Cal.) are deduced from the cDNA sequence. The amino acid numbering (AA no.) corresponds to the mature Factor VIII heavy chain. Small letters indicate tentative sequence assighnments. ND = not detected.

Source	Peptide	AA no.	Sequence	M_r Obs.	M_r Cal.
FVIII(HC:1-740)	A	725-740	DISAYLLSKNNA-IEPR	1804.9	1805.0
FVIII(HC:1-740)	B	717-720	DYYE	-	589.6
FVIII(HC:1-740)	D	519-524	DGPTKS	(604.1)	604.6
		717-720	DYYE	-	589.6
		721-724	DSYE	-	513.5
FVIII(HC:1-729)	C	725-729	DISAY	-	568.6
FVIII(HC:1-729)	D	717-720	DYyE	ND	589.6
FVIII(HC:1-729)	E	519-524	DGPTKS	ND	604.6
FVIII(HC:1-729)	F	721-724	DSYE	ND	513.5

The peak marked A in the map of FVIII(HC:1-740) corresponds to the C-terminal AspN peptide. Sequencing of the "shoulder" (retention time 27.5 min, Fig. 3) of the peak from FVIII(HC:1-729) eluting just before peptide A, did not show the sequence of the C-terminal peptide (not shown). In accordance with the results of the LysC peptide maps, this shows that only FVIII(HC:1-740) contains the full-length A2-domain. The peptide from FVIII(HC:1-729) marked C on Fig. 3 showed the sequence corresponding to amino acid 725-729, showing that FVIII(HC:1-729) have C-terminus at Tyr729. The peaks marked E, D and F in the map of FVIII(HC:1-729) corresponds to amino acid 717-720, 519-524, and 721-724, respectively. All peptides was also seen in the broad pead marked D from FVIII-(HC:1-740). The corresponding broad peak from FVIII(HC1-720) was not sequenced. FVIII(HC:1-740) did also contain amino acid 717-720 in the peak marked B. Like in the LysC peptide mapping, this is probably due to partial sulfation of Tyr

718 or Tyr 719. Because AspN cleaves between amino acid 720 and 721, a specific C-terminal peptide is not observed for FVIII(HC:1-720).

Affinity purification of the C-terminal peptide from FVIII(HC:1-720) heavy chain

The C-terminal LysC peptides from the heavy chain of FVIII(HC:1-720) were purified by anhydrotrypsin affinity chromatography. Anhydrotrypsin is a catalytical inactive derivative of trypsin with the ability of binding peptides with C-terminal Lys or Arg (Ishii and Kumazaki, (1988) in Methods in Protein Sequence Analysis. (B. Witman-Liebold, ed.) pp. 156-163, Springer Verlag, Berlin). Because the results of the peptide maps indicated, that the FVIII(HC:1-720) did not have Arg as the C-terminal amino acid of the heavy chain, the C-terminal peptide was recovered from the flow-through fraction from a LysC digest of reduced and alkylated heavy chain from FVIII(HC:1-720) loaded on an anhydrotrypsin column. Table III shows the amino acid sequence of this C-terminal peptide, clearly confirming that FVIII(HC:1-720) have C-terminus at Glu720.

Table III. Sequence analysis of the peptide from a LysC digest of heavy chain from FVIII(HC:1-740) not bound to anhydrotrypsin agarose. It was not possible to obtain a mass by mass spectrometry. The amino acid numbering (AA no.) corresponds to the mature Factor VIII heavy chain.

			M_r	
Source	AA no.	Sequence	Obs.	Cal.
FVIII(HC:1-720)	714-720	NTGDYYE	-	813.9

Specific activity

The specific activity of FVIII(HC:1-740), FVIII(HC:1-729), and FVIII(HC:1-720) was measured by both a chromogenic assay and a one-stage clotting assay (see Table IV, below).

Table IV. Specific activity of the FVIII(HC:1-740), FVIII(HC:1-729) and FVIII(HC:1-720) determined in a clot-assay (ACL analysis) and a chromogenic assay (Coatest). For each sample by 8-12 activity analyses were made. The protein concentration was measured by 2-3 amino acid analysis of each sample. The values are ± standard deviation.

Sample	Specific activity	
	Clot-assay (10^3 U/mg)	Chromogenic assay (10^3 U/mg
Unmodified FVIII	10.0 ± 2.5	8.5 ± 1.3
Modified "middle" FVIII	9.6 ± 3.0	8.2 ± 1.2
Modified "lower" FVIII	5.1 ± 1.2	10.0 ± 1.4

The specific activity of all the Factor VIII forms was approximately 1.0×10^4 U/mg as determined by the chromogenic assay. FVIII(HC:1-740) and FVIII(HC:1-729) also have the same specific activity within the experimental error as determined by the clotting assay. However, FVIII(HC:1-720) have a specific activity a factor two lower as determined in the clotting assay.

Inhibition of Factor VIII activity by a monoclonal antibody

The monoclonal antibody (F25-IgG) used for separation of FVIII(HC:1-740) from FVIII(HC:1-729) and FVIII(HC:1-720) was used for inhibition of activity of plasma Factor VIII and the three recombinant Factor VIII forms as measured by a

chromogenic assay and a one-stage clotting assay (Fig. 4). The antibody inhibits the activity of FVIII(HC:1-740) and plasma Factor VIII at a similar degree as measured in the clotting assay, but was not able to inhibit in the chromogenic assay. In the clotting assay the time of clotting of Factor VIII deficient plasma is measured. In the chromogenic assay, the Factor VIII form is incubated with Factor IXa, Factor X and phospholipid for 15 minutes before the Factor Xa substrate assay is added. This means, that any differences in affinity of for example thrombin to Factor VIII would be masked by the long incubation time in the chromogenic assay but not in the clotting assay. As expected, the inhibitory effect of the antibody was not seen for FVIII(HC:1-729) and FVIII(HC:1-720).

Time-study of thrombin activation of Factor VIII forms

Thrombin activation of plasma Factor VIII and the three recombinant Factor VIII forms was evaluated in a time-study by SDS-PAGE (Fig. 5) and a clotting assay (Fig. 6). Plasma Factor VIII, FVIII(HC:1-740) and FVIII(HC:1-729) is activated at similar rates, while FVIII(HC:1-720) is activated more slowly. At SDS-PAGE (Fig. 5) it is seen, that the heavy chain bands dissapeares within 5 to 10 min after thrombin is added for FVIII(HC:1-740), FVIII(HC:1-729), and plasma Factor VIII, while thrombin cleavage of FVIII(HC:1-720) requires at least 15 min before the heavy chain band dissapeares. Similary, the bands corresponding to the A1 and A2-domains from the activated heavy chain and the activated light chain (marked LC' on Fig. 5) is observed later for FVIII(HC:1-720) compared with the other Factor VIII forms. It is difficult to compare the dissapearance of the light chain band due to the prescence of free light chain in the plasma Factor VIII preparation. At Fig. 6 it is seen that plasma Factor VIII, FVIII(HC:1-740) and FVIII-(HC:1-729) all reaches maximum activity after 1 to 2 min of incubation with thrombin, and have similar profiles of the activity curves. FVIII(HC:1-720) is activated more slowly, ie. the activity curve shows a broad maximum after 3 to 5

min. As expected from the differences in specific activity, FVIII(HC:1-720) is less active in the clotting assay compared with the other Factor VIII forms.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): 2880
(G) TELEPHONE: 44448888
(H) TELEFAX: 44493256
(I) TELEX: 37304

(ii) TITLE OF INVENTION:
(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
(EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: DK

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 720 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Human Factor VIII

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1 5 10 15

Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
20 25 30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
35 40 45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
50 55 60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65 70 75 80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
85 90 95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
100 105 110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
115 120 125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
130 135 140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145 150 155 160

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
165 170 175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
180 185 190

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
195 200 205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
210 215 220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225 230 235 240

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
245 250 255

Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
260 265 270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
275 280 285

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
290 295 300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305 310 315 320

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
325 330 335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
340 345 350

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
355 360 365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
370 375 380

Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385 390 395 400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
405 410 415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
420 425 430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
435 440 445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
450 455 460

Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465 470 475 480

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
485 490 495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
500 505 510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
515 520 525

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
530 535 540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545 550 555 560

Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
565 570 575

Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
580 585 590

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
595 600 605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
610 615 620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625 630 635 640

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
645 650 655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
660 665 670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
675 680 685

Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
690 695 700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705 710 715 720

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 729 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Human Factor VIII

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1 5 10 15

Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
20 25 30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
35 40 45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
50 55 60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65 70 75 80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
85 90 95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
100 105 110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
115 120 125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
130 135 140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145 150 155 160

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
165 170 175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
180 185 190

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
195 200 205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
210 215 220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225 230 235 240

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
245 250 255

Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
260 265 270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
275 280 285

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
290 295 300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305 310 315 320

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
325 330 335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
340 345 350

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
355 360 365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
370 375 380

Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385 390 395 400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
405 410 415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
420 425 430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
435 440 445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
450 455 460

Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465 470 475 480

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
485 490 495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
500 505 510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
515 520 525

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
530 535 540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545 550 555 560

Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
565 570 575

Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
580 585 590

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
595 600 605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
610 615 620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625 630 635 640

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
645 650 655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
660 665 670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
675 680 685

Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
690 695 700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705 710 715 720

Asp Ser Tyr Glu Asp Ile Ser Ala Tyr
725

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 740 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Human Factor VIII

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1 5 10 15

Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
20 25 30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
35 40 45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
50 55 60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65 70 75 80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
85 90 95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
100 105 110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
115 120 125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
130 135 140

Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145 150 155 160

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
165 170 175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
180 185 190

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
195 200 205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
210 215 220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225 230 235 240

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
245 250 255

Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
260 265 270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
275 280 285

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
290 295 300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305 310 315 320

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
325 330 335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
340 345 350

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
355 360 365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
370 375 380

Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385 390 395 400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
405 410 415

Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
420 425 430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
435 440 445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
450 455 460

Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465 470 475 480

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
485 490 495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
500 505 510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
515 520 525

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
530 535 540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545 550 555 560

Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
565 570 575

Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
580 585 590

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
595 600 605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
610 615 620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625 630 635 640

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
645 650 655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
660 665 670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
675 680 685

Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
690 695 700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705 710 715 720

Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
725 730 735

Ile Glu Pro Arg
740

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 684 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Human Factor VIII

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: C-terminal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Glu Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr
1 5 10 15

Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr
20 25 30

Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg
35 40 45

His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr Gly Met Ser
50 55 60

Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly Ser Val Pro
65 70 75 80

Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe Thr
85 90 95

Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly Leu Leu Gly
100 105 110

Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val Thr Phe Arg
115 120 125

Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser Leu Ile Ser Tyr
130 135 140

Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg Lys Asn Phe Val Lys
145 150 155 160

Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys Val Gln His His Met Ala
165 170 175

Pro Thr Lys Asp Glu Phe Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp
180 185 190

Val Asp Leu Glu Lys Asp Val His Ser Gly Leu Ile Gly Pro Leu Leu
195 200 205

Val Cys His Thr Asn Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr
210 215 220

Val Gln Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser
225 230 235 240

Trp Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn
245 250 255

Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His Ala
260 265 270

Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met Ala Gln
275 280 285

Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn
290 295 300

Ile His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys
305 310 315 320

Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu
325 330 335

Thr Val Glu Met Leu Pro Ser Lys Ala Gly Ile Trp Arg Val Glu Cys
340 345 350

Leu Ile Gly Glu His Leu His Ala Gly Met Ser Thr Leu Phe Leu Val
355 360 365

Tyr Ser Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His Ile
370 375 380

Arg Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln Trp Ala Pro
385 390 395 400

Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr
405 410 415

Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile
420 425 430

Ile His Gly Ile Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser Leu
435 440 445

Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp
450 455 460

Gln Thr Tyr Arg Gly Asn Ser Thr Gly Thr Leu Met Val Phe Phe Gly
465 470 475 480

Asn Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile
485 490 495

Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg Ser
500 505 510

Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys Ser Met
515 520 525

Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala
530 535 540

Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro Ser Lys Ala
545 550 555 560

Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val Asn
565 570 575

Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr Met Lys Val
580 585 590

Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr Ser Met Tyr
595 600 605

Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly His Gln Trp Thr
610 615 620

Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe Gln Gly Asn Gln Asp
625 630 635 640

Ser Phe Thr Pro Val Val Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg
645 650 655

Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg
660 665 670

Met Glu Val Leu Gly Cys Glu Ala Gln Asp Leu Tyr
675 680

CLAIMS

What is claimed is:

1. A Factor VIII polypeptide comprising a heavy chain having an amino acid sequence corresponding to the amino acid sequence of the N-terminal part of full length Factor VIII and a light chain having an amino acid sequence corresponding to the amino acid sequence of the C-terminal part of full length Factor VIII wherein the heavy chain is shorter than the A1-A2 domain of full length Factor VIII.

2. A Factor VIII polypeptide as claimed in claim 1 wherein the heavy chain comprises the amino acids 1-729 of the heavy chain of full length Factor VIII.

3. A Factor VIII polypeptide as claimed in claim 1 wherein the heavy chain comprises the amino acids 1-720 of the heavy chain of full length Factor VIII.

4. A Factor VIII polypeptide as claimed in any of claims 1 to 3 comprising a light chain having an amino acid sequence corresponding to amino acids 1649-2332 of the C terminal of full length Factor VIII.

5. A Factor VIII polypeptide as claimed in any of claims 1 to 3 comprising a light chain having an amino acid sequence corresponding to amino acids 1690-2332 of the C terminal full length Factor VIII.

6. A method for preparing a Factor VIII polypeptide comprising a heavy chain having an amino acid sequences corresponding to the amino acid sequence of the N-terminal part of full length Factor VIII and a light chain having an amino acid sequence corresponding to the amino acid sequence of the C-terminal part of full length Factor VIII wherein all or a part of the heavy chain is shorter than the A1-

A2 domain of full length Factor VIII wherein a Factor VIII polypeptide comprising the full A1-A2 domain of full length Factor VIII is treated with a protease.

7. A pharmaceutical preparation comprising a Factor VIII polypeptide comprising a heavy chain having an amino acid sequences corresponding to the amino acid sequence of the N-terminal part of full length Factor VIII and a light chain having an amino acid sequence corresponding to the amino acid sequence of the C-terminal part of full length Factor VIII wherein all or a part of the Faktor VIII has a heavy chain shorter than the A1-A2 domain of full length Factor VIII in admixture with a parenterally acceptable vehicle or excipient.

8. Use of a Factor VIII polypeptide comprising a heavy chain having an amino acid sequences corresponding to the amino acid sequence of the N-terminal part of full length Factor VIII and a light chain having an amino acid sequence corresponding to the amino acid sequence of the C-terminal part of full length Factor VIII wherein all or a part of the Factor VIII has a heavy chain shorter than the A1-A2 domain of full length Factor VIII for the preparation of a pharmaceutical preparation.

9. Use as claimed in Claim 7 wherein a Factor VIII polypeptide comprising a heavy chain having an amino acid sequences corresponding to the amino acid sequence of the N-terminal part of full length Factor VIII and a light chain having an amino acid sequence corresponding to the amino acid sequence of the C-terminal part of full length Factor VIII wherein all or a part of the Factor VIII has a heavy chain shorter than the A1-A2 domain of full length Factor VIII for the preparation of a pharmaceutical preparation for the prevention or treatment of diseases caused by absence or deficiency of Factor VIII in a subject.

10. A method for preventing or treating diseases caused by absence or deficiency of Factor VIII in a subject comprising administering to the subject a pharmaceutically active amount of a Factor VIII polypeptide comprising a heavy

chain having an amino acid sequences corresponding to the amino acid sequence of the N-terminal part of full length Factor VIII and a light chain having an amino acid sequence corresponding to the amino acid sequence of the C-terminal part of full length Factor VIII wherein all or a part of the Factor VIII has a heavy chain shorter than the A1-A2 domain of full length Factor VIII in admixture with a pharmaceutically acceptable vehicle or excipient.

12. A method of preparing a pharmaceutical preparation comprising a Factor VIII polypeptide comprising a heavy chain having an amino acid sequences corresponding to the amino acid sequence of the N-terminal part of full length Factor VIII and a light chain having an amino acid sequence corresponding to the amino acid sequence of the C-terminal part of full length Factor VIII wherein all or a part of the Factor VIII has a heavy chain shorter than the A1-A2 domain of full length Factor VIII with pharmaceutically acceptable vehicle and/or exhipient and forming a suitable dosis form of the pharmaceutical preparation.

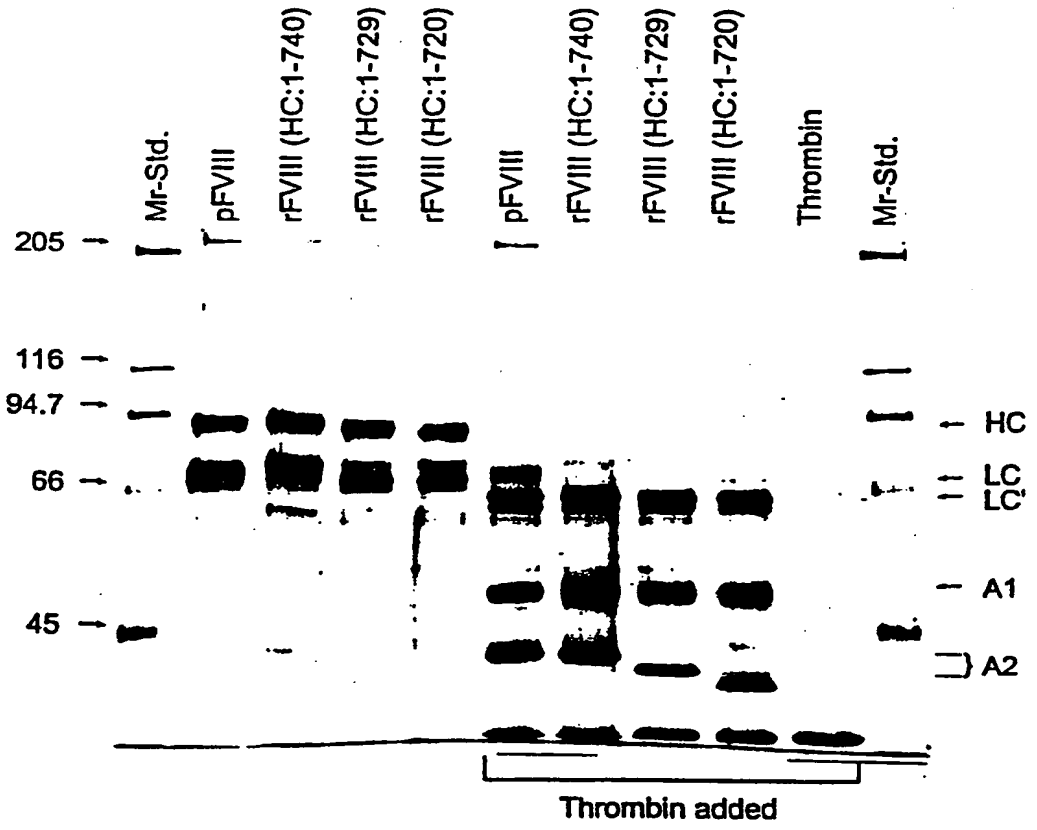

Mr-Std.
pFVIII
rFVIII (HC:1-740)
rFVIII (HC:1-729)
rFVIII (HC:1-720)
pFVIII
rFVIII (HC:1-740)
rFVIII (HC:1-729)
rFVIII (HC:1-720)
Thrombin
Mr-Std.
205
116
94.7
66
45
HC
LC
LC'
A1
A2
Thrombin added

Fig. 1

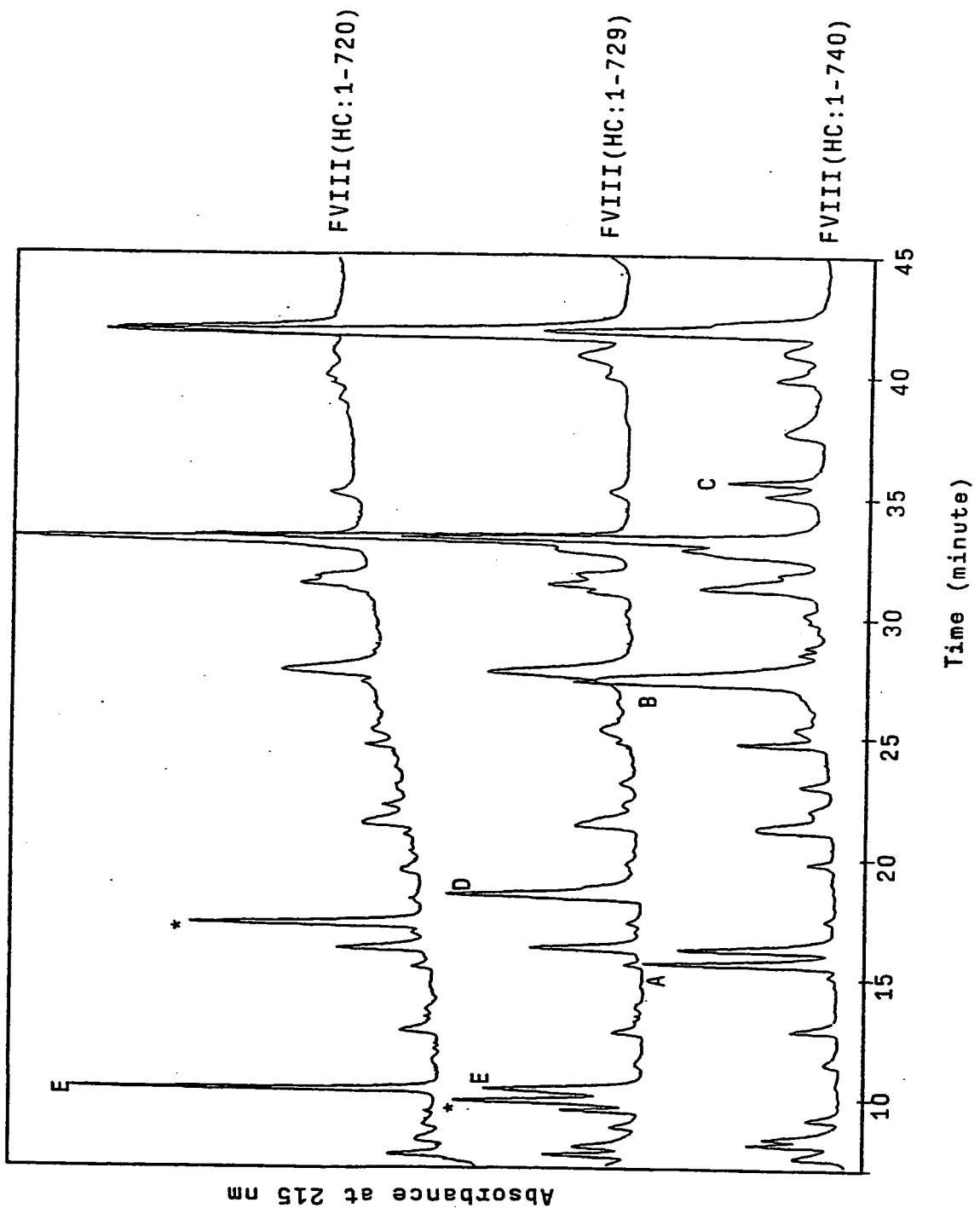


Fig. 2

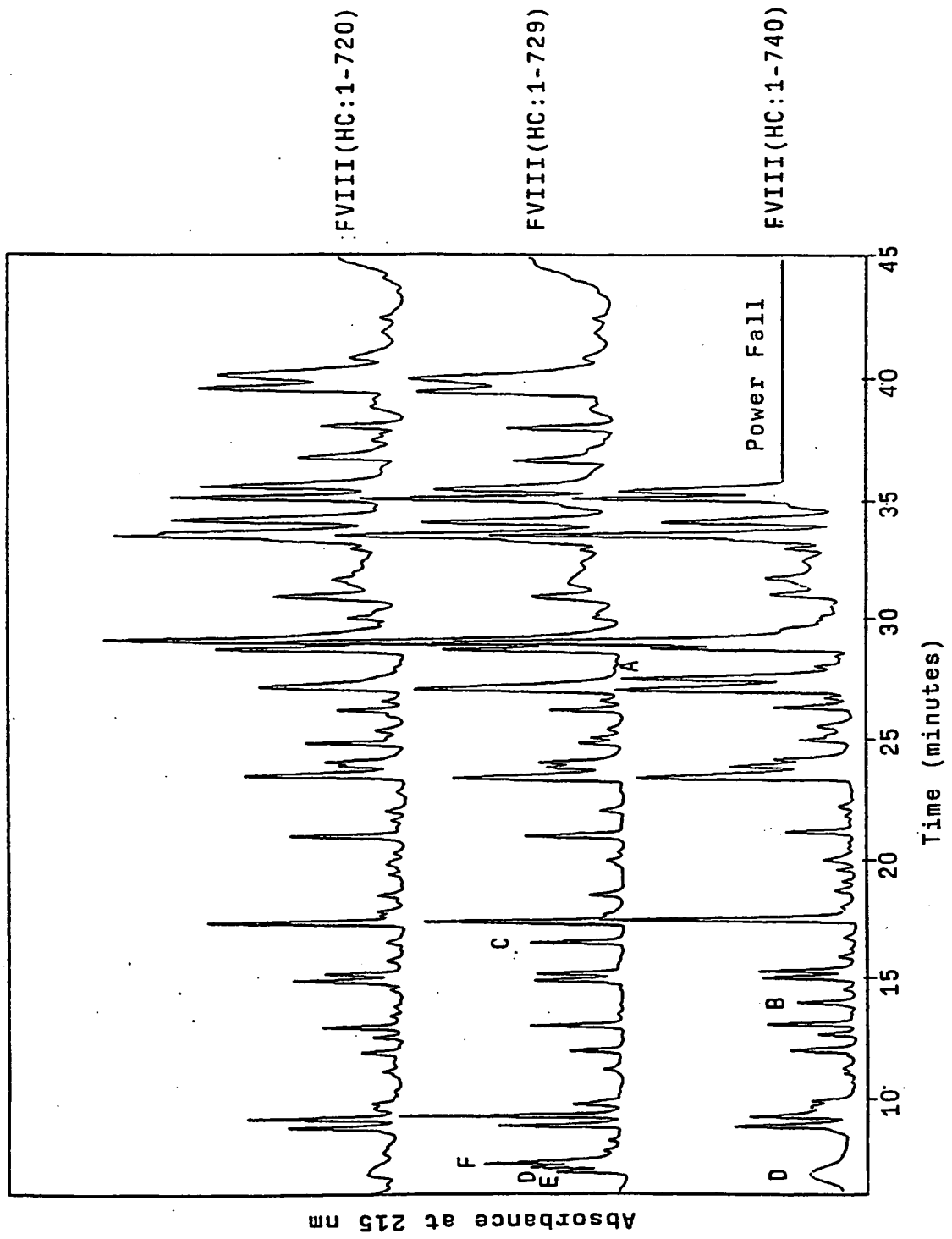

FVIII(HC:1-720)
FVIII(HC:1-729)
FVIII(HC:1-740)
Power Fall
A
B
C
D
D
E
F
10
15
20
25
30
35
40
45
Time (minutes)
Absorbance at 215 nm

Fig. 3

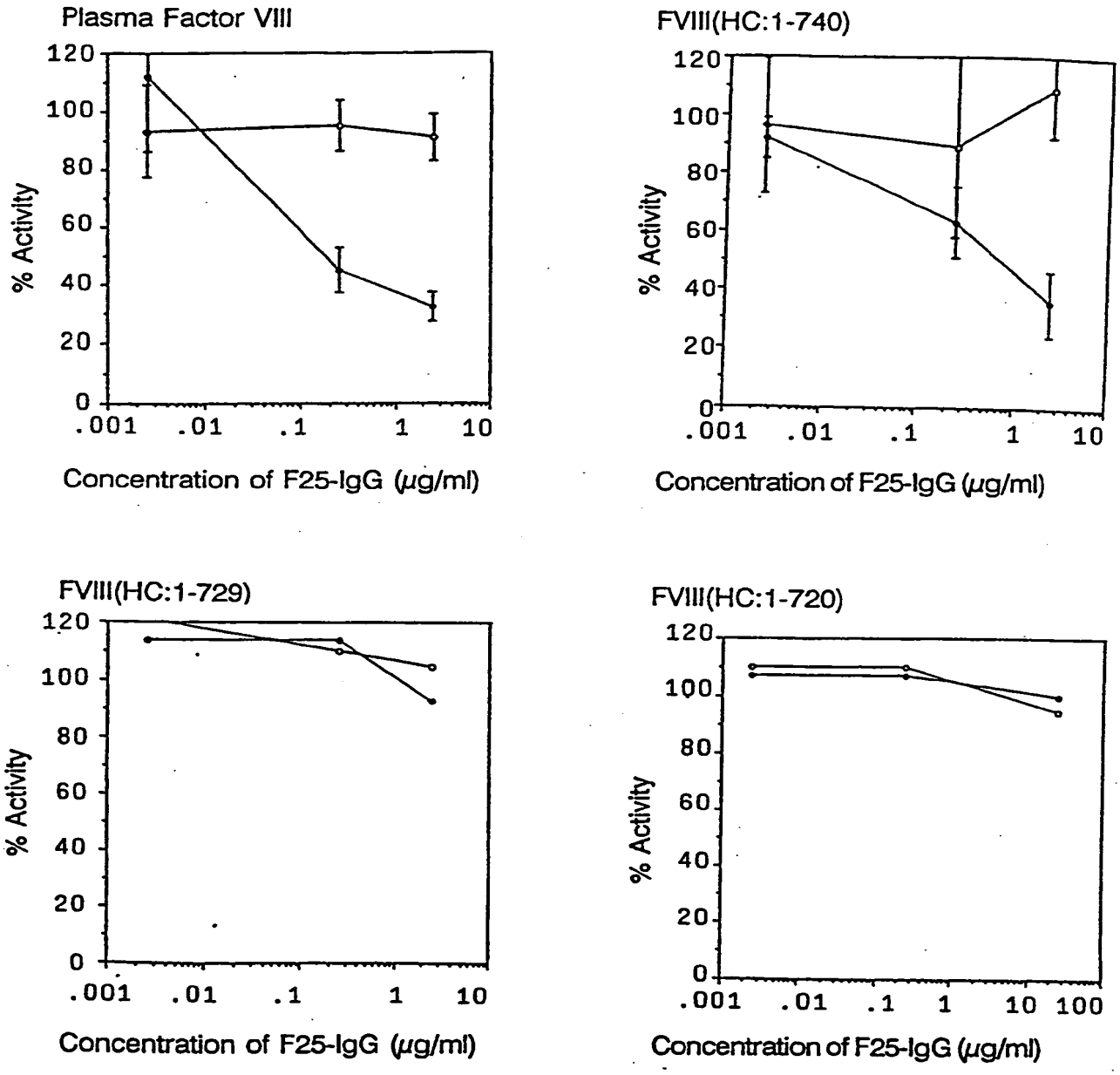
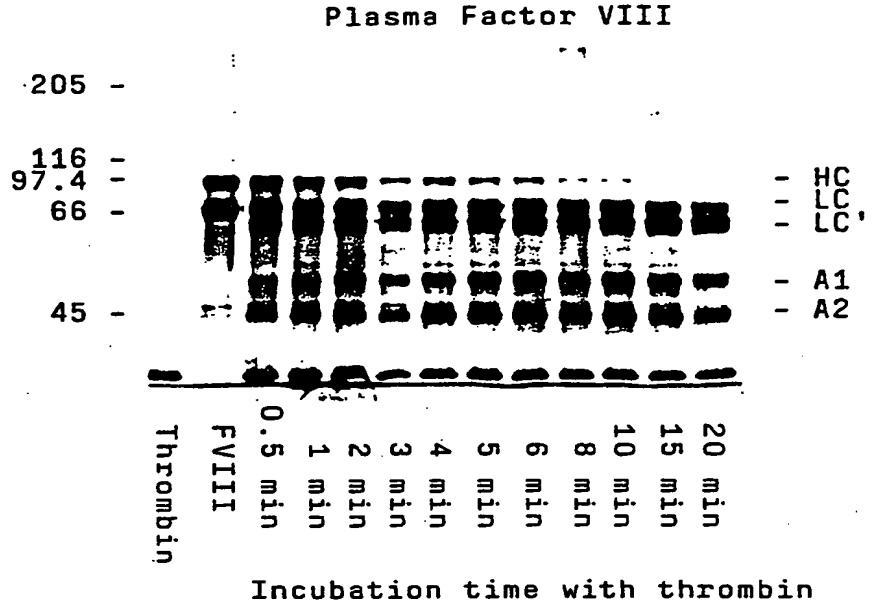


Fig. 4



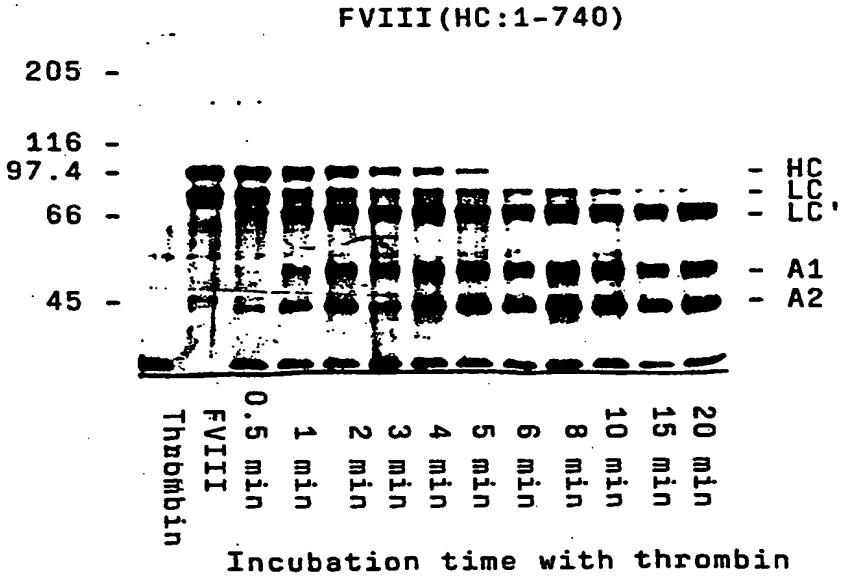
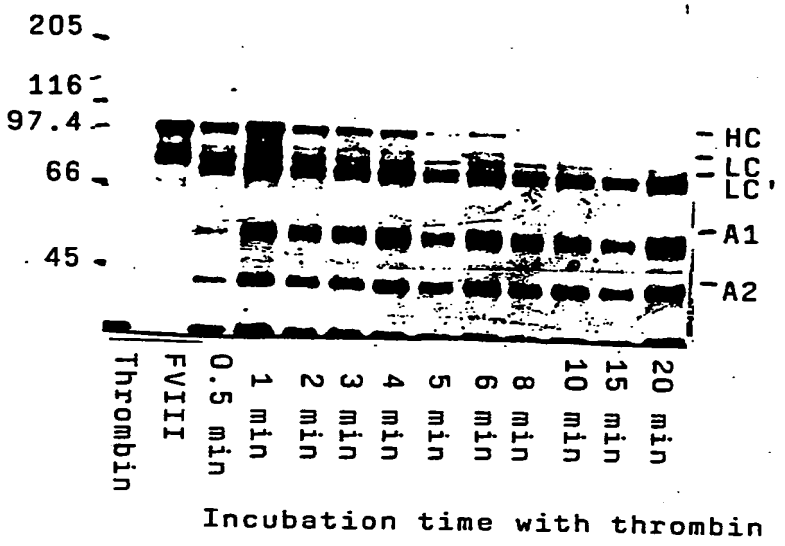


Fig. 5A

FVIII(HC:1-729)



FVIII(HC:1-720)

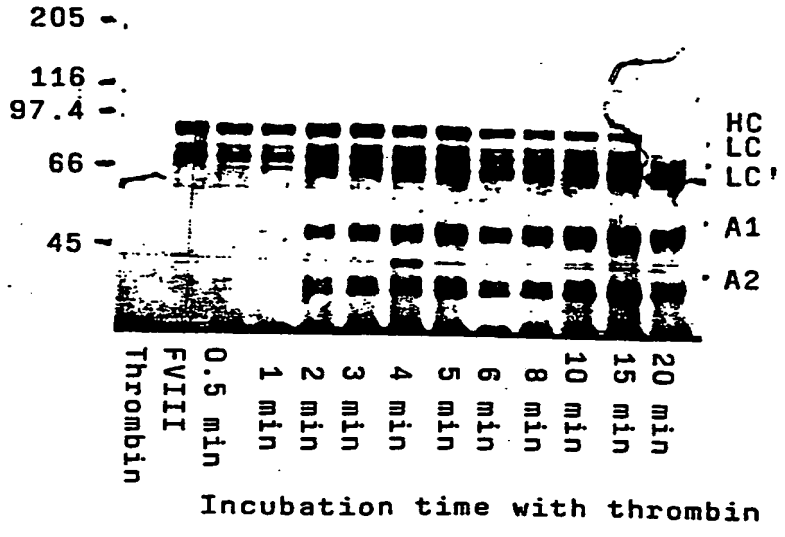


Fig. 5B

Thrombin activation of factor VIII forms

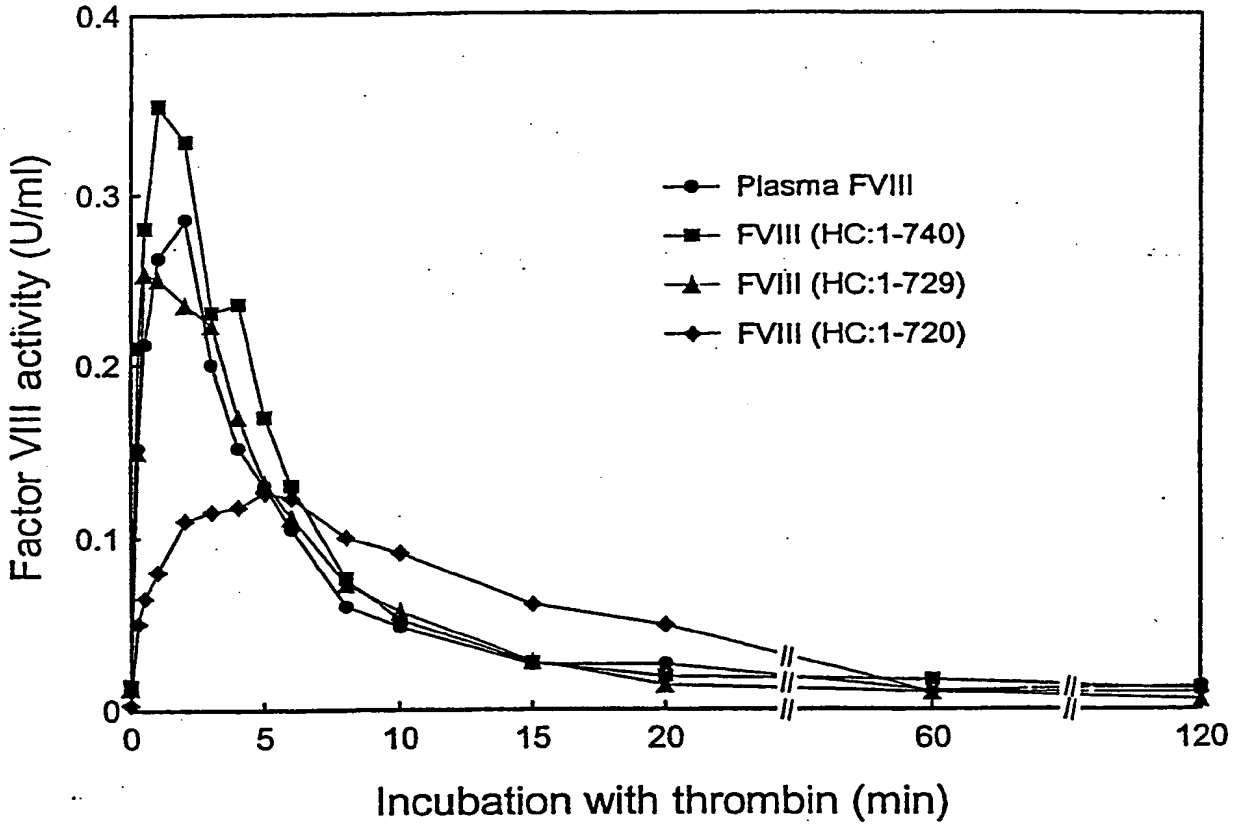


Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00423

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/755
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, WPIL, US PATENTS FULLTEXT DATABASES

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP, A1, 0294910 (GIST-BROCADES N.V.), 14 December 1988 (14.12.88), page 3, line 10; page 7, line 47 - line 49; page 13, line 7 - line 8, page 18, table I; page 19, table II; see claims 7 and 21 --	1-9,12
X	WO, A1, 8707144 (GENETICS INSTITUTE, INC.), 3 December 1987 (03.12.87) --	1,6-9,12

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

- * Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" erlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 February 1995	24 -02- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00423

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Dialog Information Services, file 5, Biosis, Dialog accession No.10906084, Biosis accession No. 97106084, Kjalke M. et al: "Function of recombinant factor VIII with heavy chain consisting of amino acid 1-740, 1-729 and 1-720", Blood 82 (10 Suppl. 1), 1993, 60A -- --------	1-9,12

INTERNATIONAL SEARCH REPORT

Information on patent family members

31/12/94

International application No.
PCT/DK 94/00423

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0294910	14/12/88	AU-A- 1809788	04/01/89
		IL-A- 86693	24/06/94
		US-A- 5171844	15/12/92
		WO-A- 8809813	15/12/88
WO-A1- 8707144	03/12/87	AU-B- 609043	26/04/91
		AU-A- 7486887	22/12/87
		EP-A- 0270618	15/06/88
		JP-T- 63503357	08/12/88

INTERNAT[redacted] SEARCH REPORT

International application No.
PCT/DK 94/00423

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 10
because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1 (iv): Methods for treatment of the human or animal body by therapy.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.